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(54) Title: SIMPLE CATALYTIC DNA BIOSENSORS FOR IONS BASED ON COLOR CHANGES

(57) Abstract: Disclosed are compositions and methods for the sensitive and selective detection of ions using nucleic acid enzymes and DNA modified microparticles.

SIMPLE CATALYTIC DNA BIOSENSORS FOR IONS BASED ON COLOR CHANGES

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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BACKGROUND

Many metals pose a risk as environmental contaminants. A well-known example is lead. Low level lead exposure can lead to a number of adverse health effects, with as many as 9-25% of pre-school children presently at risk. The level of lead in the blood considered toxic is ≥ 10 μg/dL (480 nM). Current methods for lead analysis, such as atomic absorption spectrometry, inductively coupled plasma mass spectrometry, and anodic stripping voltammetry, often require sophisticated equipment, sample pre-treatment, and skilled operators.

Simple, rapid, inexpensive, selective and sensitive methods that permit real time detection of Pb^{2+} and other metal ions are very important in fields such as environmental monitoring, clinical toxicology, wastewater treatment, and industrial process monitoring. Furthermore, methods are needed for monitoring free or bioavailable, instead of total, metal ions in industrial and biological systems.

Many fluorescent chemosensors, including fluorophore-labeled organic chelators (Rurack, *et al.*, 2000; Hennrich *et al.*, 1999; Winkler *et al.*, 1998; Oehme & Wolfbeis, 1997) and peptides (Walkup & Imperiali, 1996; Deo & Godwin, 2000; Pearce *et al.*, 1998), have been developed for metal ion detection. These ion sensors are usually composed of an ion-binding motif and a fluorophore. Metal detection using these fluorescent chemosensors relies on the modulation of the fluorescent properties of the fluorophore by the metal-binding event. Detection limits on the level of micromolar and even nanomolar concentrations have been achieved for heavy metal ions including Zn^{2+}, Cu^{2+}, Hg^{2+}, Cd^{2+} and Ag^{+}. The design and synthesis of a chemosensor that exhibits highly selective and sensitive binding of the metal ion of choice in aqueous solution is still a big challenge, although the metal binding and the

fluorescent moieties of the sensor can be systematically varied to achieve desired properties. Although fluorescence spectroscopy is a technique well suited for detecting very small concentrations of analytes, a fluorometer is required to generate and detect the emitted signal. Thus, the need for expensive instrumentation and complicated operation procedures make this method impractical for applications such as household use, field testing or small clinic testing.

Nucleic acid molecules have previously been adapted to sense the presence of nucleic acids and to detect gene mutations from inherited diseases or chemical damages. In recent years, the molecular recognition and catalytic function of nucleic acids have been extensively explored. This exploration has lead to the development of aptamers and nucleic acid enzymes.

Aptamers are single-stranded oligonucleotides derived from an *in vitro* evolution protocol called systematic evolution of ligands by exponential enrichment (SELEX). Nucleic acid aptamers have been isolated from random sequence pools and can selectively bind to non-nucleic acid targets, such as small organic molecules or proteins, with affinities as high as 10^{-14} M (Uphoff *et al.*, 1996; Famulok, 1999). Most aptamers undergo a conformational change when binding their cognate ligands. With this property, several DNA and RNA aptamers have been engineered to sense L-adenosine or thrombin through an internally labeled fluorescent reporter group (Jhaveri *et al.*, 2000). Here, the conformational change in the aptamer upon binding leads to a change in fluorescence.

Nucleic acid enzymes are nucleic acid molecules that catalyze a chemical reaction. *In vitro* selection of nucleic acid enzymes from a library of 10^{14}-10^{15} random nucleic acid sequences offers considerable opportunity for developing enzymes with desired characteristics (Breaker & Joyce, 1994; Breaker, 1997). Compared with combinatorial searches of chemo- and peptidyl-sensors, *in vitro* selection of DNA/RNA is capable of sampling a larger pool of sequences, amplifying the desired sequences by polymerase chain reactions (PCR), and introducing mutations to improve performance by mutagenic PCR.

Allosteric ribozymes (or aptazymes), which combine the features of both aptamer and catalytic RNA, also hold promises for sensing small molecules (Potyrailo *et al.*, 1998; Koizumi *et al.*, 1999; Robertson & Ellington, 1999, 2000). Their

reactivity is modulated through the conformational changes caused by the binding of small organic molecules to an allosteric aptamer domain. Therefore, the signal of ligand binding can be transformed into a signal related to chemical reaction.

Divalent metal ions can be considered as a special class of cofactors controlling the activity of nucleic acid enzymes. The reaction rate of the nucleic acid enzymes depends on the type and concentration of the metal ion in solution. Several RNA and DNA enzymes obtained through *in vitro* selection are highly specific for Cu^{2+}, Zn^{2+}, and Pb^{2+}, with metal ion requirements on the level of micromolar concentrations (Breaker & Joyce, 1994; Pan & Uhlenbeck, 1992; Carmi *et al.*, 1996; Pan et al., 1994; Cuenoud & Szotak, 1995; Li *et al.*, 2000; Santoro *et al.*, 2000).

A variety of methods have been developed for assembling metal and semiconductor colloids into nanomaterials. These methods have focused on the use of covalent linker molecules that possess functionalities at opposing ends with chemical affinities for the colloids of interest. One of the most successful approaches to date, (Brust *et al.*, (1995)), involves the use of gold colloids and well-established thiol adsorption chemistry (Bain & Whitesides, (1989); Dubois & Nuzzo (1992)). In this approach, linear alkanedithiols are used as the particle linker molecules. The thiol groups at each end of the linker molecule covalently attach themselves to the colloidal particles to form aggregate structures. The drawbacks of this method are that the process is difficult to control and the assemblies are formed irreversibly. Methods for systematically controlling the assembly process are needed if the materials properties of these structures are to be exploited fully.

The potential utility of DNA for the preparation of biomaterials and in nanofabrication methods has been recognized. Researchers have focused on using the sequence-specific molecular recognition properties of oligonucleotides to design impressive structures with well-defined geometric shapes and sizes. Shekhtman *et al.*, (1993); Shaw & Wang, (1993); Chen *et al.*, (1989); Chen & Seeman, (1991); Smith and Feigon (1992); Wang *et al.*, (1993); Chen *et al.*, (1994); Marsh *et al.*, (1995); Mirkin (1994); Wells (1988); Wang *et al.*, (1991). However, the theory of producing DNA structures is well ahead of experimental confirmation. Seeman et al., New J. Chem., 17, 739-755 (1993).

Agglutation assays are well known for the detection of varoius analytes. The

basic principle of an agglutination assay is the formation of clumps (agglutination or aggregation) of small particles coated with a binding reagent when exposed to a multi-valent binding partner specific for the binding reagent. Particles routinely used in agglutation assays include, for example, latex particles, erythrocytes (RBCs), or bacterial cells (often stained to make the clumps visible). Typically, a binding reagent, such as an antibody, is attached to the particles. A sample thought to contain the analyte of interest is contacted with a suspension of such coated particles. If the analyte is present, cross linking of the particles occurs due to bond formation between the antibodies on the particles and the analyte in the sample. Such binding results in the agglutation of the particles which can be detected either visually or with the aid of simple instrumentation. In an alternative protocol, an antigen is attached to the particles and the presence of an antibody specific for the antigen detected in the sample.

More recently, metal, semiconductor and magnetic particles have been used in aggregation assays. For example, particles comprising metal (e.g., gold, silver, copper and platinum), semiconductor (e.g., CdSe, CdS, and CdS or CdSe coated with ZnS) and magnetic (e.g., ferromagnetite) colloidal materials have been described. Other particle types include ZnS, ZnO, TiO_2, AgI, AgBr, HgI_2, PbS, PbSe, ZnTe, CdTe, In_2S_3, In_2Se_3, Cd_3P_2, Cd_3As_2, InAs, and GaAs.

Mirkin et al. (U.S. Patent Number 6,361,944) describes aggregation assays for detecting a nucleic acid in a sample. This method involves incubating a sample thought to contain a nucleic acid with particles having oligonucleotides attached to the surface (particle-oligonucleotide conjugates). The oligonucleotides on each particle have an oligonucleotide complementary to the sequences of at least two portions of the nucleic acid. Alternatively, at least two types of particles having oligonucleotides attached may be used. The oligonucleotides on the first type of particles having a sequence complementary to one portion of the nucleic acid and the oligonucleotides on the second type of particles have a sequence complementary to a second portion of the sequence of the nucleic acid. The incubation takes place under conditions effective to allow hybridization of the oligonucleotides on the particles with the nucleic acid in the sample. Such a hybridization results in an aggregation of the particles. This produces a color change which may be detected visually or with

simple instrumentation. For example, the aggregation of gold particles results in a color change from red to purple (Mirkin (U.S. Patent Number 6,361,944)).

Methods of detection based on observing such a color change with the naked eye are cheap, fast, simple, robust (the reagents are stable) and do not require specialized or expensive equipment. This makes such methods particularly suitable for use in applications such as the detection of lead in paint or heavy metals in water.

BRIEF SUMMARY

The present invention provides a method of detecting the presence of an ion in a sample. A new class of DNA enzyme-based biosensor for ions is provided. This combines the high selectivity of DNA enzymes with the convenience of particle aggregation-based detection. Such selectivity and convenience provides for semi-quantitative and quantitative detection of ions over a concentration range of several orders of magnitude.

The sample may be any solution that may contain an ion (before or after pre-treatment). The sample may contain an unknown concentration of an ion and may contain other ions. For example, the sample may be paint that is tested for lead content. The sample may be diluted yet still remains a sample. The sample may be obtained from the natural environment, such as a lake, pond, or ocean, an industrial environment, such as a pool or waste stream, a research lab, common household, or a biological environment, such as blood.

In one embodiment, the invention provides a nucleic acid enzyme, a substrate and particles. The nucleic acid enzyme, a substrate and particles are provided as, or form an aggregate. The method for detection of the ion comprises contacting the aggregate with the ion wherein the nucleic acid enzyme causes cleavage of the substrate in the presence of the ion. The method for detection of the ion may be performed in the presence of other ions.

In a preferred embodiment, the particles are gold particles and the presence of the ion in detected by observing a color change resulting from a breakdown of the aggregate.

In another aspect, the invention provides kits for the detection of an ion.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Selection scheme for RNA-cleaving deoxyribozymes. **FIG. 1A.** (SEQ ID NO: 12) Starting pool of random-sequenced DNAs, engineered to contain two substrate-binding domains. Each member of the pool contains a 5'-terminal biotin (encircled B), a single embedded ribonucleotide (rA) and a 40-nucleotide random sequence domain (N40). **FIG. 1B.** Selective amplification scheme for isolation of DNA that catalyzes the metal cofactor (Co^{2+} or Zn^{2+}) dependent cleavage of an RNA phosphodiester.

FIG. 2. (SEQ ID NOS 13-23, respectively, in order of appearance) Sequence classes of the cloned Zn-DNA. The numbers on the left are the clone-numbers randomly assigned to the sequences during the cloning and sequencing process. The highly conserved sequences (Region-20nt) are in bold. The covariant nucleotides are underlined. The 5'- and the 3'-primer binding sequences are shown in italic.

FIG 3. (SEQ ID NOS 24-42, respectively, in order of appearance) Sequence classes of the cloned Co-DNA. The clone-numbers are listed on the left. The 5' and the 3' primer binding sequences are in italic.

FIG. 4. (SEQ ID NOS 43-70, respectively, in order of appearance) Sequence alignment of the N40 region of the reselected Zn-DNAs. The wild-type sequence is listed on the top, followed by the reselected Zn-DNA sequences. Only the point mutations are shown for the reselected sequences, while the nucleotides that are identical to the wild type at the corresponding positions are omitted. Numbers listed on the left are clone-numbers. The rate constants (k_{obs}) of several reselected Zn-DNA in 100 μM Zn^{2+} are shown on the right.

FIG. 5. (SEQ ID NOS 1 & 2) Proposed secondary structure of the Zn(II)-dependent *trans*-cleaving deoxyribozyme.

FIG. 6. Sequences and proposed secondary-structures of several RNA-cleaving deoxyribozymes. **FIG. 6A** (SEQ ID NOS 71 & 72) **and FIG. 6B** (SEQ ID NOS 73 & 74). The deoxyribozyme selected using Mg^{2+} or Pb^{2+} as cofactor (Breaker & Joyce, 1994, 1995). **FIG. 6C** (SEQ ID NOS 75 & 76) **and FIG. 6D** (SEQ ID NOS 77 & 78). The $1\bar{0}23$ and the $\bar{8}17$ deoxyribozymes selected in Mg^{2+} to cleave all-RNA substrate (Santoro & Joyce, 1997). **FIG. 6E** (SEQ ID NOS 79 & 80). A

deoxyribozyme selected using L-histidine as cofactor. **FIG. 6F** (SEQ ID NOS 81 & 82). The 17E deoxyribozyme selected in Zn^{2+}. In each structure, the upper strand is the substrate and the lower strand is the enzyme. Arrows identify the site of RNA transesterification.

FIG. 7. Comparison of G3 deoxyribozyme with class II Co-DNA. **FIG. 7A.** (SEQ ID NO: 83) The predicted secondary structure of the G3 deoxyribozyme (Geyer & Sen, 1997). X represents variable sequences. The boxed region was also found in class II Co-DNA. **FIG. 7B.** (SEQ ID NO: 84) The minimal structure motif of the class II Co-DNA predicted by *mfold* program. The arrows indicate the cleavage sites.

FIG. 8. Figure showing an assay protocol for particle based DNA assay for Pb^{++}.

DETAILED DESCRIPTION

The invention described herein represents a new class of DNA enzyme-based biosensor for ions. It combines the high selectivity of DNA enzymes with the convenience of particle aggregation-based detection. Such selectivity and convenience provides for semi-quantitative and quantitative detection of ions over a concentration range of several orders of magnitude. In a preferred embodiment, the aggregation-based detection domain is decoupled from the ion-recognition/catalysis domain, and therefore the sensitivity and selectivity of this system may be manipulated by a careful choice of particles and aggregation method, and by performing *in vitro* selection of ion-binding domains to not only keep sequences reactive with the ion of choice, but also remove sequences that also respond to other ions.

Nucleic Acid Enzymes

A growing number of nucleic acid enzymes have been discovered or developed showing a great diversity in catalytic activity (Table 1 and Table 2). Many if not all of the enzymes are dependent on one or more ion cofactors. *In vitro* selection may be used to "enhance" selectivity and sensitivity for a particular ion. Such enzymes find particular utility in the compositions and methods of the present invention. For example, nucleic acid enzymes that catalyze molecular association

(ligation, phophorylation, and amide bond formation) or dissociation (cleavage or transfer) are particularly useful.

In preferred embodiments, a nucleic acid enzyme that catalyzes the cleavage of a nucleic acid in the presence of an ion is used. The nucleic acid enzyme may be RNA (ribozyme), DNA (deoxyribozyme), a DNA/RNA hybrid enzyme, or a peptide nucleic acid (PNA) enzyme. PNAs comprise a polyamide backbone and the bases found in naturally occurring nucleosides and are commercially available from, e.g., Biosearch, Inc. (Bedford, Mass.).

Ribozymes that may be used in the present invention include, but are not limited to, group I and group II introns, the RNA component of the bacterial ribonuclease P, hammerhead, hairpin, hepatitis delta virus and Neurospora VS ribozymes. Also included are *in vitro* selected ribozymes, such as those isolated by Tang and Breaker (2000).

One limitation of using a ribozyme is that they tend to be less stable than deoxyribozymes. Thus, in preferred embodiments, the nucleic acid enzyme is a deoxyribozyme. Preferred deoxyribozymes include those shown in FIG. 6A-6F and deoxyribozymes with extended chemical functionality (Santoro *et al.*, 2000).

Table 1.

Reactions catalyzed by ribozymes that were isolated from *in vitro* selection experiments.

Reaction	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/k_{uncat} [a]	Reference
Phosphoester centers				
Cleavage	0.1	0.03	10^5	Vaish, 1998
Transfer	0.3	0.02	10^{13}	Tsang, 1996
Ligation	100	9	10^9	Ekland, 1995
Phosphorylation	0.3	40	$>10^5$	Lorsch, 1994
Mononucleotide polymerization	0.3	5000	$>10^7$	Ekland, 1996
Carbon centers				
Aminoacylation	1	9000	10^6	Illangasekare, 1997
Aminoacyl ester hydrolysis	0.02	0.5	10	Piccirilli, 1992
Aminoacyl transfer	0.2	0.05	10^3	Lohse, 1996
N-alkylation	0.6	1000	10^7	Wilson, 1995
S-alkylation	4×10^{-3}	370	10^3	Wecker, 1996
Amide bond cleavage	1×10^{-5}		10^2	Dai, 1995
Amide bond formation	0.04	2	10^5	Wiegand, 1997
Peptide bond formation	0.05	200	10^6	Zhang, 1997
Diels-Alder cycloaddition	>0.1	>500	10^3	Tarasow, 1997
Others				
Biphenyl isomerization	3×10^{-5}	500	10^2	Prudent, 1994
Porphyrin metallation	0.9	10	10^3	Conn, 1996

[a] Reactions catalyzed by ribozymes that were isolated from *in vitro* selection experiments. kcat/kuncat is the rate enhancement over uncatalyzed reaction.

Table 2.
Deoxyribozymes isolated through *in vitro* selection.

Reaction	Cofactor	$k_{max}(min^{-1})^a$	k_{cat}/k_{uncat}	Reference
RNA transesterification	Pb^{2+}	1	10^5	Breaker, 1994
	Mg^{2+}	0.01	10^5	Breaker, 1995
	Ca^{2+}	0.08	10^5	Faulhammer, 1997
	Mg^{2+}	10	$>10^5$	Santoro, 1997
	None	0.01	10^8	Geyer, 1997
	L-histidine	0.2	10^6	Roth, 1998
	Zn^{2+}	~40	$>10^5$	Li, J., 2000
DNA cleavage	Cu^{2+}	0.2	$>10^6$	Carmi, 1996
DNA ligation	Cu^{2+} or Zn^{2+}	0.07	10^5	Cuenod, 1995
DNA phosphorylation	Ca^{2+}	0.01	10^9	Li, Y., 1999
5',5'-pyrophophate formation	Cu^{2+}	5×10^{-3}	$>10^{10}$	Li, Y., 2000
Porphyrin metalation	None	1.3	10^3	Li, Y., 1996

[a] k_{max} is the maximal rate constant obtained under optimized conditions.

An advantage of ribozymes and deoxyribozymes is that they may be produced and reproduced using biological enzymes and appropriate templates. However, the present invention is not limited to ribozymes and deoxyribozymes. Nucleic acid enzymes that are produced by chemical oligosynthesis methods are also included. Thus, nucleic acids including nucleotides containing modified bases, phosphate, or sugars may be used in the compositions and methods of the present invention. Modified bases are well known in the art and include inosine, nebularine, 2-aminopurine riboside, N^7-denzaadenosine, and O^6-methylguanosine (Earnshaw & Gait, 1998). Modified sugars and phosphates are also well known and include 2'-deoxynucleoside, abasic, propyl, phosphorothioate, and 2'—O-allyl nucleoside (Earnshaw & Gait, 1998). DNA/RNA hybrids and PNAs may be used in the compositions and methods of the present invention. The stability of PNAs and

relative resistance to cellular nucleases make PNA enzymes amenable to *in vivo* applications.

In certain embodiments, the substrate for the nucleic acid enzyme and the enzyme itself are contained in the same nucleic acid strand. Such enzymes are *cis*-acting enzymes. Examples include the Zn^{2+}-dependent deoxyribozymes (Zn-DNA) created in Example 2 (FIG. 1A and FIG. 2).

In preferred embodiments, the nucleic acid enzyme cleaves a nucleic acid strand that is separate from the strand comprising the enzyme (*trans*-acting). One advantage of utilizing *trans*-activity is that, after cleavage, the product is removed and additional substrate may be cleaved by the enzymatic strand. A preferred nucleic acid enzyme is 5'-CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT-3' (17E; FIG. 5; SEQ ID NO:1). The corresponding preferred substrate to 17E is 5'-ACTCACTATrAGGAAGAGATG-3' (17DS; FIG. 5; SEQ ID NO:2), where rA denotes a single ribonucleotide.

It may be beneficial to use directed mutation to change one or more properties of a nucleic acid enzyme or its substrate. Using 17E and 17DS as an example, one may wish to alter the avidity of the two arms of the hybridized enzyme and substrate. The "arms" are those areas displaying Watson-Crick basepairing in FIG. 5. To alter avidity, one may increase or decrease the length of the arms. Increasing the length of the arms increases the number of Watson-Crick bonds, thus increasing the avidity. The opposite is true for decreasing the length of the arms. Decreasing the avidity of the arms facilitates the removal of substrate from the enzyme, thus allowing faster enzymatic turnover.

Another method of decreasing avidity includes creating mismatches between the enzyme and the substrate. Alternatively, the G-C content of the arms may be altered. Of course, the effect of any directed change should be monitored to ensure that the enzyme retains its desired activity, including ion sensitivity and selectivity. In light of the present disclosure, one of skill in the art would understand how to monitor for a desired enzymatic activity. For example, to ensure that the mutated enzyme maintained sensitivity and selectivity for Pb^{2+}, one would test to determine if the mutated enzyme remained reactive in the presence of lead (sensitivity) and maintained its lower level of activity in the presence of other ions (selectivity).

The nucleic acid enzyme is sensitive and selective for a single ion. The ion may be any anion, for example, arsenate (AsO_4^{3-}), or cation. The ion may be monovalent, divalent, trivalent, or polyvalent. Examples of monovalent cations include K^+, Na^+, Li^+, Tl^+, NH_4^+ and Ag^+. Examples of divalent cations include Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Hg^{2+}, Pt^{2+}, Ra^{2+}, Ba^{2+}, UO_2^{2+} and Sr^{2+}. Examples of trivalent cations include Co^{3+}, Cr^{3+}, and lanthanide ions (Ln^{3+}). Polyvalent cations include Ce^{4+}, Cr^{6+}, spermine, and spermidine. The ion detected by the biosensor also includes ions having a metal in a variety of oxidation states. Examples include K(I), Na(I), Li(I), Tl(I), Ag(I), Hg(I), Mg(II), Ca(II), Mn(II), Co(II), Ni(II), Zn(II), Cd(II), Pb(II), Hg(II), Pt(II), Ra(II), Ba(II), Sr(II), Co(III), Cr(III), Ln(III), Ce(IV), Cr(VI) and U(VI).

The biosensors of the present invention may be used to monitor contaminants in the environment; in such a case preferred ions are those that are toxic to living organisms, *e.g.*, Ag^+, Pb^{2+} and Hg^{2+}.

Often the nucleic acid enzymes that have activity with one ion also have at least some activity with one or more other ions. Such multi-sensitive enzymes may still be used in the compositions and methods of the present invention. However, it should be understood that use of a multi-sensitive enzyme may lead to uncertainty as to which of the ions is present. In such cases, measuring the rate of enzymatic activity, using serial dilutions, or using an array of nucleic acid enzymes may be helpful in deciphering which ion is present.

In vitro Selection of Nucleic Acid Enzymes

Many nucleic acid enzymes that are dependent on ions, particularly metal ions, for activity are known in the art (Breaker & Joyce, 1994; Pan & Uhlenbeck, 1992; Cuenoud & Szostak, 1995; Carmi *et al.*, 1996; Li *et al.*, 2000; Santoro *et al.*, 2000). In light of the present disclosure, one of skill in the art would understand how to utilize a known nucleic acid enzyme in the methods and biosensors of the present invention. Furthermore, the present invention may include a nucleic acid enzyme created by *in vitro* selection. Methods of *in vitro* selection of nucleic acid enzymes are known in the art and described herein.

In vitro selection is a technique in which RNA or DNA molecules with certain functions are isolated from a large number of sequence variants through multiple cycles of selection and amplification (Joyce, 1994; Chapman *et al.*, 1994). The concept of *in vitro* selection of catalytic RNA molecules was first introduced in the late 1980's. Since then, it has been widely applied to obtain ribozymes with maximized activities or novel catalytic abilities, and to identify oligonucleotides (called aptamers) that bind to certain proteins or small molecules with high affinity. The process for aptamers selection is sometimes referred as systematic evolution of ligands by exponential enrichment (SELEX)(Tuerk & Gold, 1990).

The first catalytic DNA (deoxyribozyme) was isolated by Breaker and Joyce in 1994 through *in vitro* selection. This deoxyribozyme is able to catalyze phosphodiester cleavage reaction in the presence of Pb^{2+}. Unlike RNA-based catalysts, DNA molecules with catalytic functions have not been encountered in nature, where DNA exists primarily as base-paired duplex and serves mainly as the carrier of genetic information. The identification of DNA molecules with catalytic functions further demonstrated the power of *in vitro* selection.

In vitro selection is typically initiated with a large collection of randomized sequences. A typical DNA or RNA library for selection contains 10^{13}-10^{16} sequence variants. The construction of a completely randomized pool is accomplished by chemical synthesis of a set of degenerated oligonucleotides using standard phosphoramidite chemistry. The 3'-phosphoramidite compounds of four nucleosides (A, C, G, and T) are premixed before being supplied to an automated DNA synthesizer to produce oligonucleotides. By controlling the ratio of four phosphoroamidites, the identity at each nucleotide position can be either completely random, i.e. with equal chance for each base, or biased toward a single base. Other strategies for creating a randomized DNA library include applying mutagenic polymerase chain reaction (PCR) and template-directed mutagenesis (Tsang and Joyce, 1996; Cadwell and Joyce, 1992, 1994). For the purpose of *in vitro* selection of functional RNA molecules, the randomized DNA library is converted to an RNA library through *in vitro* transcription.

In vitro selection takes advantage of a unique property of RNA and DNA, i.e., the same molecule can possess both genotype (coding information) and phenotype

(encoded function). The DNA or RNA molecules in the randomized library are screened simultaneously. Those sequences that exhibit a desired function (phenotype) are separated from the inactive molecules. Usually the separation is performed through affinity column chromatography, being linked to or released from a solid support, gel electrophoresis separation, or selective amplification of a tagged reaction intermediate. The genotype of the active molecules are then copied and amplified, normally through polymerase chain reaction (PCR) for DNA or isothermal amplification reaction for RNA. Mutations can be performed with mutagenic PCR to reintroduce diversity to the evolving system. These three steps —selection, amplification and mutation, are repeated, often with increasing selection stringency, until sequences with the desired activity dominate the pool.

Novel nucleic acid enzymes isolated from random sequences *in vitro* have extended the catalytic repertoire of RNA and DNA far beyond what has been found in nature. The selected ribozymes are capable of catalyzing a wide range of reactions at both phosphate and non-phosphate centers (Table 1). The reactions that are catalyzed by deoxyribozymes are less diverse, compared with the ribozymes (Table 2). However, the catalytic rate (k_{cat}) of most deoxyribozymes is comparable to that of the ribozymes catalyzing the same reaction. In certain cases, the catalytic efficiency (k_{cat}/K_m) of nucleic acid enzymes even exceeds that of the protein enzymes.

In vitro selection can be used to change the ion specificity or binding affinity of existing ribozymes, or to obtain nucleic acid enzymes specific for desired ions. For example, *in vitro*-selected variants of the group I intron (Lehman & Joyce, 1993) and the RNase P ribozyme (Frank & Pace, 1997) have greatly improved activity in Ca^{2+}, which is not an active metal ion cofactor for native ribozymes. The Mg^{2+} concentration required for optimal hammerhead ribozyme activity has been lowered using *in vitro* selection to improve the enzyme performance under physiological conditions (Conaty *et al.*, 1999; Zillman et al., 1997). Breaker and Joyce have isolated several RNA-cleaving deoxyribozymes using Mg^{2+}, Mn^{2+}, Zn^{2+}, or Pb^{2+} as the cofactor (Breaker & Joyce, 1994, 1995). Only the sequence and structure of the Pb^{2+}-dependent and the Mg^{2+}-dependent deoxyribozymes were reported (FIG. 6A and 6B). Other examples of metal-specific RNA/DNA enzymes obtained through *in vitro* selection include a Pb^{2+}-specific RNA-cleaving ribozyme (called leadzyme)(Pan &

Uhlenbeck, 1992), a Cu^{2+}-specific DNA-cleaving deoxyribozyme (Carmi *et al.*, 1996), and a DNA ligase active in Zn^{2+} and Cu^{2+} (Cuonod & Szostak, 1995).

Often nucleic acid enzymes developed for a specific metal ion by *in vitro* selection will have activity in the presence of other metal ions. For example, 17E deoxyribozyme was developed by *in vitro* selection for activity in the presence of Zn^{2+}. Surprisingly, the enzyme showed greater activity in the presence of Pb^{2+} than Zn^{2+}. Thus, although produced in a process looking for Zn^{2+}-related activity, 17E may be used as a sensitive and selective sensor of Pb^{2+}.

To produce nucleic acid enzymes with greater selectivity, a negative selection step may be included in the process. For Example, Pb^{2+}-specific deoxyribozymes may be isolated using a similar selection scheme as for the selection of Co^{2+}- and Zn^{2+}-dependent DNA enzymes described in Example 2. In order to obtain deoxyribozymes with high specificity for Pb^{2+}, negative-selections may be carried out in addition to the positive selections in the presence of Pb^{2+}.

For negative selection, the DNA pool is selected against a "metal soup", which contains various divalent metal ions (e.g. Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, etc.). Those sequences that undergo self-cleavage in the presence of divalent metal ions other than Pb^{2+} are then washed off the column. The remaining sequences are further selected with Pb^{2+} as the cofactor. Pb^{2+}-dependent deoxyribozymes with different affinities for Pb^{2+} can be obtained by controlling the reaction stringency (Pb^{2+} concentration). Besides the oligonucleotide sequences discussed here, other sequences shall be useful in the practicing of this invention. Such sequences are described in U.S Patent Application Serial Number 09/605558, filed June 27th, 2000, the contents of which are incorporated by this reference.

DNA Coated Particles

Methods of making metal, semiconductor and magnetic particles are well-known in the art. Methods of making ZnS, ZnO, TiO_2, AgI, AgBr, HgI_2, PbS, PbSe, ZnTe, CdTe, In_2S_3, In_2Se_3, Cd_3P_2, Cd_3As_2, InAs, and GaAs particles are also known. See, e.g., Mirkin et al. U.S. Patent Number 6,361,944. Suitable particles are also commercially available from, e.g., Amersham Biosciences, (Piscataway, NJ) (gold) and Nanoprobes, Inc. (Yaphank, NY) (gold).

In addition, other types of particles may be used in the present invention. For example, polystyrene latex particles or latex particles containing dye may be used. The critical requirement is that a detectable change must occur upon aggregation/ aggregate breakdown. In addition, the composition of the particles must be such that the particles do not interfere with the cleavage of the substrate by the nucleic acid enzyme in the presence of the metal ion.

Gold colloidal particles are preferred for use in detecting metal ions. Gold colloidal particles have high extinction coefficients for the bands that give rise to their intense colors. These colors vary with particle size, concentration, interparticle distance, and extent of aggregation and shape of the aggregates, making these materials particularly useful for calorimetric assays. For instance, hybridization of oligonucleotides attached to gold particles with oligonucleotides and nucleic acids results in an immediate color change visible to the naked eye (see, e.g., Mirkin et al. U.S. Patent Number 6,361,944).

Gold particles, oligonucleotides or both are functionalized in order to attach the oligonucleotides to the particles. Such methods are known in the art. For instance, oligonucleotides functionalized with alkanethiols at their 3'-termini or 5'-termini readily attach to gold particles. See Whitesides (1995). See also, Mucic *et al.* (1996) (describes a method of attaching 3' thiol DNA to flat gold surfaces; this method can be used to attach oligonucleotides to particles). The alkanethiol method can also be used to attach oligonucleotides to other metal, semiconductor and magnetic colloids and to the other particles listed above. Other functional groups for attaching oligonucleotides to solid surfaces include phosphorothioate groups (see, e.g., U.S. Pat. No. 5,472,881 for the binding of oligonucleotide-phosphorothioates to gold surfaces), substituted alkylsiloxanes (see, e.g. Burwell (1974) and Matteucci and Caruthers (1981) for binding of oligonucleotides to silica and glass surfaces, and Grabar *et al.*, (1995) for binding of aminoalkylsiloxanes and for similar binding of mercaptoaklylsiloxanes). Oligonucleotides terminated with a 5' thionucleoside or a 3' thionucleoside may also be used for attaching oligonucleotides to solid surfaces. Gold particles may be attached to oligonucleotides using biotin-labeled oligonucleotides and streptavidin-gold conjugate colloids; the biotin-streptavidin interaction attaches the colloids to the oligonucleotide. Shaiu et al., (1993). The following references

describe other methods which may be employed to attached oligonucleotides to particles: Nuzzo et al., (1987) (disulfides on gold); Allara and Nuzzo (1985) (carboxylic acids on aluminum); Allara and Tompkins (1974) (carboxylic acids on copper); Iler, (1979) (carboxylic acids on silica); Timmons and Zisman, (1965) (carboxylic acids on platinum); Soriaga and Hubbard, (1982) (aromatic ring compounds on platinum); Maoz and Sagiv, (1987) (silanes on silica).

Each particle will have a plurality of oligonucleotides attached to it. As a result, each particle-oligonucleotide conjugate can bind to a plurality of oligonucleotides or nucleic acids having the complementary sequence.

Oligonucleotides of defined sequences are used for a variety of purposes in the practice of the invention. Methods of making oligonucleotides of a predetermined sequence are well-known. See, e.g., Sambrook *et al.*, (1989) and F. Eckstein (1991). Solid-phase synthesis methods are preferred for both oligoribonucleotides and oligodeoxyribonucleotides (the well-known methods of synthesizing DNA are also useful for synthesizing RNA). Oligoribonucleotides and oligodeoxyribonucleotides can also be prepared enzymatically.

Biosensors

Described herein are nucleic acid enzymes that are dependent on the presence of a specific ion for activity. Using particle labeling, it is possible to measure enzymatic activity, and hence ion concentration, without the need of complex instrumentation. These qualities make the compositions of the present invention excellent for use in biosensors for detecting ions.

Many biosensors utilizing nucleic acids are known in the art. For example, biosensors using aptamers have been developed for detecting molecules such as thrombin or adenosine (Potyrailo *et al.*, 1999; Lee & Walt, 2000). In light of the present disclosure, one of ordinary skill in the art would know how to modify the nucleic acid biosensors to include nucleic acid enzymes.

In a simple embodiment, a biosensor of the present invention comprises a nucleic acid enzyme, a cleavable substrate for the nucleic acid enzyme, and particles labeled with oligoribonucleotides complementary to at least two portions of the cleavable substrate. Preferably, the substrate is modified, for example, by extension

of the 3' and 5' ends by a number of bases which act as "sticky end" for annealing to the complementary DNA strand on the particles. Modification of the substrate allows complexes comprising of substrate linked particles to be formed without inhibiting the nucleic acid enzyme/ cleavable substrate interaction. However, where the substrate contains regions not critical for interaction with the nucleic acid enzyme, modification may not be necessary.

In a method using this embodiment the nucleic acid enzyme, cleavable substrate, and labeled particles are combined to form aggregated complexes. Such aggregates are contacted with a sample suspected of containing an ion to which the enzyme is sensitive. In the presence of the ion, the enzyme cleaves the substrate causing a break-up of the aggregates. An example of such an assay protocol is shown in Figure 8. In some embodiments, heating of the aggregates to a temperature above the "melting point" of the complex may be necessary. In such an embodiment, the presence of the ion allows the enzyme to cleave the substrate, preventing "re-aggregation" of the complex. However, in certain embodiments, heating is not required (see, for example, Example 8).

A change in the aggregation state of the particles may be detected by observing an color change which accompanies the change in state. For example, the a breakdown of aggregation of gold particles results in a color change from purple to red. Furthermore, the amount of substrate cleavage depends on the concentration of the ion. A low concentration of ion results in only partial cleavage of the substrate, producing a mixture of single particles and aggregates with different degree of aggregations. Hence, this simple color test can be used as a semi-qualitative or qualitative way to detect an ion, for example, Pb^{2+}. The color difference can be amplified to improve the sensitivity of the method. For example, this may be achieved by spotting the resulting solution onto an alumina TLC plate. Different degrees of color change are produced, depending on the amount of ion present. Alternatively, a quantitative assay can be achieved by measuring the optical spectra of the assay mixture.

The detectable change that occurs upon a change in the aggregation state of the particles may be a color change, the formation of aggregates of the particles, or the precipitation of the aggregated particles. The color changes can be observed with

the naked eye or spectroscopically. The formation of aggregates of the particles can be observed by electron microscopy or by nephelometry. The precipitation of the aggregated particles can be observed with the naked eye or microscopically. However, changes observable with the naked eye are preferred, particularly a color change observable with the naked eye.

The observation of a color change with the naked eye can be made more readily against a background of a contrasting color. For instance, when gold particles are used, the observation of a color change is facilitated by spotting a sample of the hybridization solution on a solid white surface (such as silica or alumina TLC plates, filter paper, cellulose nitrate membranes, and nylon membranes) and allowing the spot to dry. Initially, the spot retains the color of the hybridization solution (which ranges from pink/red, in the absence of aggregation, to purplish-red/purple, if there has been aggregation). On drying, a blue spot develops if aggregation is present prior to spotting. In the absence of aggregation (e.g., because the target ion is present), the spot is pink. The blue and the pink spots are stable and do not change on subsequent cooling or heating or over time. They provide a convenient permanent record of the test. No other steps are necessary to observe the color change.

Alternatively, assay results may be visualized by spotting a sample onto a glass fiber filter (e.g., Borosilicate Microfiber Filter, 0.7 micron pore size, grade FG75, for use with gold particles 13 nm in size), while drawing the liquid through the filter. Subsequent rinsing with water washes the excess, non-hybridized probes through the filter, leaving behind an observable spot comprising the aggregates. Additional methods useful in visualizing assay results are described in Mirkin et al. U.S. Patent Number 6,361,944.

The above methods allow the ion to be detected in a variety or sample types, including a bodily fluids, and in the presence of other ions. Various techniques may be employed to improve the accuracy and precision of the above method of observation. Standards containing known amounts of ion may be assayed along side the unknown sample and the color changes compared. Alternatively, standard color charts, similar to those used with pH papers, may be provided.

Many variants of these simple embodiments are included within the scope of the invention. The specificity of the nucleic acid enzyme may be varied to allow

specific detection of a wide range of ions. In addition, the composition, size and surface properties of the particles may be optimized to obtain preferred aggregation and visual properties. Similarly, parameters such as the particle/ oligonucleotide probe coupling technology, and the length and sequence of the oligonucleotide probes and substrate may be varied to optimize the performance of the assay.

The invention also provides kits for detecting ions. In one embodiment, the kit comprises at least one container, the container holding at least one type of particles having oligonucleotides attached thereto; a cleavable substrate; and a nucleic acid enzyme. The oligonucleotides on the particles have a sequence complementary to the sequence of at least a first and a second portion of the cleavable substrate. The first and second portions of the cleavable substrate are separated by a third portion of the substrate that is cleaved by the nucleic acid enzyme in the presence of the ion. The above components may be supplied in an aggregated state.

In a second embodiment, the above kit comprises at least two types of particles having oligonucleotides attached thereto. A first type of particles has oligonucleotides which have a sequence complementary to the sequence of a first portion of the cleavable substrate. A second type of particles has oligonucleotides which have a sequence complementary to the sequence of a second portion of the cleavable substrate. The first and second portions of the cleavable substrate are separated by a third portion of the substrate that is cleaved by the nucleic acid enzyme in the presence of the ion.

When a kit is supplied, the different components of the composition may be packaged in separate containers and admixed immediately before use. Such packaging of the components separately may permit long-term storage of the active components. For example, one of more of the particles having oligonucleotides attached thereto; the cleavable substrate; and the nucleic acid enzyme are supplied in separate containers.

The reagents included in the kits can be supplied in containers of any sort such that the life of the different components are preserved and are not adsorbed or altered by the materials of the container. For example, sealed glass ampules may contain one of more of the above reagents, or buffers that have been packaged under a neutral, non-reacting gas, such as nitrogen. Ampules may consist of any suitable material,

such as glass, organic polymers, such as polycarbonate, polystyrene, *etc.*; ceramic, metal or any other material typically employed to hold similar reagents. Other examples of suitable containers include simple bottles that may be fabricated from similar substances as ampules, and envelopes, that may comprise foil-lined interiors, such as aluminum or an alloy. Other containers include test tubes, vials, flasks, bottles, syringes, or the like. Containers may have a sterile access port, such as a bottle having a stopper that can be pierced by a hypodermic injection needle. Other containers may have two compartments that are separated by a readily removable membrane that upon removal permits the components to be mixed. Removable membranes may be glass, plastic, rubber, *etc.*

The kits may also contain other reagents and items useful for detecting ions. The reagents may standard solutions containing known quantities of the ion, dilution and other buffers, pretreatment reagents, etc. Other items which may be provided as part of the kit include a solid surface (for visualizing aggregate break down) such as a TLC silica plate, microporous materials, syringes, pipettes, cuvettes and containers. Standard charts indicating the appearance of the particles in various aggregation states, corresponding to the presence of different amounts of the ion under test, may be provided.

Kits may also be supplied with instructional materials. Instructions may be printed on paper or other substrate, and/or may be supplied as an electronic-readable medium, such as a floppy disc, CD-ROM, DVD-ROM, Zip disc, videotape, audiotape, *etc.* Detailed instructions may not be physically associated with the kit; instead, a user may be directed to an internet web site specified by the manufacturer or distributor of the kit, or supplied as electronic mail.

EXAMPLES

The following examples are included to demonstrate embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the

specific embodiments that are disclosed and still obtain like or similar results without departing from the spirit and scope of the invention.

Example 1 – Preparation of Gold Particles

Gold colloids (13 nm diameter) were prepared by reduction of $HAuCl_4$ with citrate as described in Mirkin, U.S. Patent Number 6361944, Frens, (1973) and Grabar, (1995). Briefly, all glassware was cleaned in aqua regia (3 parts HCl, 1 part HNO_3), rinsed with Nanopure H_2O, then oven dried prior to use. $HAuCl_4$ and sodium citrate were purchased from Aldrich Chemical Company. Aqueous $HAuCl_4$ (1 mM, 500 mL) was brought to reflux while stirring and 38.8 mM sodium citrate (50 mL) added quickly. The solution color changed from pale yellow to burgundy. Refluxing was continued for 15 min. After cooling to room temperature, the red solution was filtered through a Micron Separations Inc. 1 micron filter. Gold colloids were characterized by UV-vis spectroscopy using a Hewlett Packard 8452A diode array spectrophotometer and by Transmission Electron Microscopy (TEM) using a JEOL 2010 transmission electron microscope.

Example 2 – In-vitro selection of an ion-dependent deoxyribozyme

A partially random DNA library was used to obtain deoxyribozymes that cleave RNA in the presence of Zn^{2+} or Co^{2+}.

Oligonucleotides

DNA oligonucleotides were purchased from Integrated DNA Technologies Inc., Coralville, IA. Sequences of the random DNA template and the primers (P1, P2 and P3) used in PCR amplifications are listed below:

P1: 5'-GTGCCAAGCTTACCG-3' (SEQ ID NO:3)

P2: 5'-CTGCAGAATTCTAATACGACTCACTATAGGAAGAGATGGCGAC-3' (SEQ ID NO:4)

P3: 5'-GGGACGAATTCTAATACGACTCACTATrA-3' (SEQ ID NO:5)

Template for random DNA pool:

5'-GTGCCAAGCTTACCGTCAC-N40-GAGATCTCGCCATCTCTTCCT ATAGTGAGTCGTATTAG-3' (SEQ ID NO:6)

Primer P1b and P3b are the 5'-biotinylated version of primers P1 and P3. Primer P1a and P3a were prepared by 5'-labeling P1 and P3 with [□-^{32}P] ATP (Amersham) and T4 polynucleotide kinase (Gibco). The DNA/RNA chimeric substrate (17DS) for *trans*-cleavage assays has the sequence *5'*-ACTCACTATrAGGAAGAGATG-3' (SEQ ID NO:2), where rA denotes a single ribonucleotide. The all-RNA substrate (17RS) with the same sequence was purchased from Dharmacon Research Inc. The *trans*-cleaving deoxyribozyme 17E has the sequence 5'-CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT-3' (SEQ ID NO:1). The deoxyribozyme named 17E1 is a variant of 17E with the sequence 5'-CATCTCTTTTGTCAGCGACTCGAAATAGTGA GT-3' (SEQ ID NO:7). All oligonucleotides were purified using denaturing polyacrylamide gel electrophoresis and desalted with the SepPak nucleic acid purification cartridges (Waters) before use.

Preparation of Random DNA Pool

The initial pool for DNA selection was prepared by template-directed extension followed by PCR amplification. The extension was carried out with 200 pmol of DNA template containing a 40-nucleotide random sequence region, and 400 pmol of primer P3b in 20× 100 μl reaction mixtures for four thermal-cycles (1 min at 92°C, 1 min at 52°C, and 1 min at 72°C). Reaction buffer also included 0.05 U/μl *Taq* polymerase (Gibco), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C), 0.01% gelatin and 0.2 mM of each dNTP. Subsequently, 1 nmol each of P1 and P3b were added to the extension product to allow four more cycles of PCR amplification. The products were precipitated with ethanol and dissolved in 0.5 mL of buffer A, which contains 50 mM HEPES (pH 7.0), 500 mM (for Zn-DNA selection) or 1 M (for Co-DNA selection) NaCl. About 20 μM EDTA was also added to the buffer to chelate trace amount of divalent metal ion contaminants.

In Vitro Selection

The random DNA pool was immobilized on a NeutrAvidin column (Pierce) by incubating with the column materials for 30 minutes. The mixture was gently vortex-mixed a few times during the incubation. The unbound DNA strands were eluted with at least 5× 100 μl of buffer A. The non-biotinylated strands of immobilized

DNA were washed off the column with 5× 100 µl of freshly prepared 0.2 M NaOH and 20 µM EDTA. The column was then neutralized with 5× 100 µl of buffer A. The cleavage reaction was carried out by incubating the immobilized single-stranded DNA containing the single ribonucleotide (rA) with 3× 20 µl of reaction buffer (buffer A plus 1 mM $ZnCl_2$ or $CoCl_2$) over 1h. The eluted DNA molecules were pooled and precipitated with ethanol. A fraction of the selected DNA was amplified in 100 µl PCR reaction with 40 pmol each of primers P1 and P2 over 10-20 thermal cycles. One tenth of the PCR product was further amplified for six cycles with 50 pmol of primers P1 and P3b. The final PCR product was ethanol precipitated and used to initiate the next round of selection. During the selection of Zn(II)-dependent deoxyribozymes (called Zn-DNA hereafter), the concentration of $ZnCl_2$ was kept constant at 100 µM in the reaction buffer for the following rounds of selection. Reaction time was gradually decreased from 1 h to 30 s within 12 rounds of selection. For the selection of Co(II)-dependent deoxyribozymes (called Co-DNA hereafter), the concentration of $CoCl_2$ was gradually decreased from 1 mM to 100 µM and the reaction time from 1 h to 1 min within 10 rounds of selection. The twelfth generation of selectively amplified Zn-DNA and the tenth generation of Co-DNA were cloned using TA-TOPO Cloning Kit (Invitrogen) and sequenced with T7 Sequenase 2.0 Quick-denatured Plasmid Sequencing Kit (Amersham).

Reselection

Based on the sequence of class I Zn-DNA or Co-DNA, partially degenerate DNA template libraries for reselection were synthesized (Integrated DNA Technology Inc., Coralville, IA) with 20% degeneracy at the N40 region. In other words, during the oligonucleotide synthesis of the N40 region, the wild type sequence was introduced at a probability of 80% at each position, while the other three nucleotides each occurred at a probability of 6.67%. The reselection pool was prepared with 10 pmol of template and 100 pmol of primers P1 and P3b using the same protocol previously described. With 10 pmol (number of molecules $S = 6 \times 10^{12}$) of partially randomized template, the statistic parameters of the DNA library used for reselection were calculated based on the following equations.

$$P(k,n,d) = [n!/(\bar{n}k)!k!]d^k(\bar{1}d)^{\bar{n}k} \qquad (1)$$

$$N(k) = [n!/(\bar{n}k)!k!]3^k \quad (2)$$

$$C(n,k) = SP(k,n,d)/N(k) \quad (3)$$

P(k,n,d) is the probability of having k mutations within n (number of randomized positions, n = 40) nucleotide positions that have been randomized at a degeneracy of d. N(k) is the number of distinct sequences that have k mutations with respect to the prototype sequence. C(n,k) is the number of copies for each sequence that has k mutations. The reselection pool was expected to contain the wild type sequence, all possible sequences with $\bar{1}8$ point mutations, and a sampling of the sequences with >8 point mutations. More than half of the population contains ≥8 point-mutations. The protocol for reselection was the same as the primary selection, except that the reaction time was decreased from 20 min to 1 min and the concentration of $ZnCl_2$ or $CoCl_2$ was decreased from 20 μM to 5 μM over six generations. The sixth generation of reselected Zn- or Co-DNA were cloned and sequenced as previously described.

Kinetic Assays of the Reselected *Cis*-cleaving DNA

The 5' ^{32}P-labeled precursor DNA for *cis*-cleavage assay was prepared by PCR-amplification of the selected DNA population or the cloned DNA plasmid with primer 1b and 3a. The double-stranded product was immobilized on a NeutrAvidin column through the biotin moiety on primer P1b. The catalytic strand of DNA was eluted off the column with 3× 20 μl freshly prepared 0.2 N NaOH and neutralized with 8 μl of 3 M sodium acetate (pH 5.3) in the presence of 50 μg/ml bovine serum albumin (Sigma). Following ethanol precipitation, the single-stranded DNA was purified on an 8% denaturing polyacrylamide gel and desalted with SepPak nucleic acid purification cartridge. Bovine serum albumin (50 μg/ml) was added to the gel-soaking buffer (0.2 M NaCl, 20 μM EDTA, 10 mM $\bar{T}$risHCl, pH 7.5) to prevent the DNA from adhering to the tube. The concentration of the DNA was determined by scintillation counting the radioactivity.

The precursor DNA was dissolved in buffer A and incubated at room temperature for 10 min before $CoCl_2$ or $ZnCl_2$ was added. The reaction was stopped with 50 mM EDTA, 90% formamide and 0.02% bromophenol blue. Reaction

products were separated on an 8% denaturing polyacrylamide gel and quantified with a Molecular Dynamic phosphorimager.

In Vitro Selection of Zn(II)- or Co(II)-dependent Deoxyribozymes

The DNA molecules capable of cleaving an RNA bond in the presence of Co^{2+} or Zn^{2+} were obtained through *in vitro* selection. The initial DNA library for selection contains ~10^{14} out of the possible 10^{24} (= 4^{40}) DNA sequences. These molecules consist of a random sequence domain of 40 nucleotides flanked by two conserved primer-binding regions. The sequence of the conserved region was designed in such a way that they could form two potential substrate-binding regions (FIG. 1A). A ribonucleic adenosine was embedded in the 5'-conserved sequence region and was intended to be the cleavage site, since an RNA bond is more susceptible than a DNA bond toward hydrolytic cleavage. The intrinsic half-life of the phosphodiester linkage in RNA at pH 7 and 25°C is estimated to be 1,000 years. The corresponding value for DNA is 200 million years.

The DNA pool was immobilized on a NeutrAvidin column through the biotin moiety on the 5' terminus of the DNA. Biotin and Avidin bind strongly with an association constant of $K_a = 10^{15}$ M^{-1}. The sequences that underwent self-cleavage in the presence of Co^{2+} or Zn^{2+} were eluted off the column, amplified and used to seed the next round of selection (FIG. 1B). The selection stringency was increased during the selection process with shorter reaction time and less available divalent metal ions. The activity of the selected Zn-DNA gradually increased until the twelfth generation and declined thereafter, while the highest activity was achieved with the tenth generation of Co-DNA. Therefore the twelfth generation of Zn-DNA and the tenth generation of Co-DNA were cloned and sequenced. The cloned sequences can be divided into different classes based on sequence similarity (FIG. 2 and FIG. 3).

Individual sequences of the cloned Zn-DNA and Co-DNA were randomly chosen and sampled for activity. Under the selection conditions (100 μM Zn^{2+}, 500 mM NaCl, 50 mM HEPES, pH 7.0, 25°C), the observed rate constants of Zn-DNAs from sequence-classes I and II were 0.1~0.2 min^{-1}, while class III sequences were less active, with k_{obs} around 0.02 min^{-1}. The cleavage rate of the initial pool was 2×10^{-7} min^{-1}. Therefore, a 10^5~10^6 fold increase in cleavage rate has been achieve for Zn-

DNA selection. The cleavage rates of all the randomly picked Co-DNA sequences were <0.02 min^{-1} under the conditions for Co-DNA selections (100 μM Co^{2+}, 1 M NaCl, 50 mM HEPES, pH 7.0, 25°C). Interestingly, even in the buffer (1 M NaCl, 50 mM HEPES, pH 7.0) alone, the class II Co-DNA exhibited similar activity as in the presence of 100 μM Co^{2+} or Zn^{2+}.

Clone #5 of Zn-DNA (Zn-5) and clone #18 of Co-DNA (Co-18) showed relatively high activity, as well as high frequency of occurrence, within their lineages. The k_{obs} were 0.17 min^{-1} for Zn-5 (in 100 μM Zn^{2+}) and 0.02 min^{-1} for Co-18 (in 100 μM Co^{2+}). The sequences of Zn-5 and Co-18 were partially randomized (see Material and Methods for details) and subjected to reselection in order to further improve the reactivity and metal-binding affinity, and to explore the sequence requirement of the conserved catalytic motif. Based on equations (1)~(3), the reselection pool was expected to contain the wild type sequence, all possible sequences with ~18 point mutations, and a sampling of the sequences with >8 point mutations. More than half of the population should contain ≥8 point mutations. Six rounds of reselection were carried out with ~520 μM Zn^{2+}or Co^{2+}, however the activity of the reselected DNA was similar to the activity of the wild type sequences. Sequencing of the Zn-DNA from both the initial selection and reselection revealed a highly conserved sequence region. Therefore the lack of activity improvement after reselection likely reflects a sequence pool dominated by a few highly reactive sequences.

Sequence Alignment and Structure Analysis of Zn-DNA

The sequences of thirty individual clones of initially selected Zn-DNA can be divided into three major classes based on sequence similarity. Differences among members of each class were limited to a few point mutations (FIG. 2). A highly conserved sequence region of 20 nt, 5'-$TX_1X_2X_3AGCY_1Y_2Y_3TCGAAATAGT$-3' (SEQ ID NO:8) (Region-20nt), was observed in all but one sequence albeit at different locations. The sequences of 5'-$X_1X_2X_3$-3' and 3'-$Y_3Y_2Y_1$-5' are complimentary and covariant, indicating that they form base pair with each other:

5'-$X_1X_2X_3$-3'

3'-$Y_3Y_2Y_1$-5'

The secondary structures of the sequenced Zn-DNA were predicted using Zuker's *DNA mfold* program (see http://mfold.wustl.edu/~folder/dna/form1.cgi) through minimization of folding energy. The most stable structures predicted for those containing Region-20nt all contained a similar structure motif. This common motif consists of a pistol-shaped three-way helical junction formed by a 3 bp hairpin, an 8 bp hairpin and a double helix linking to the rest of the molecule. The 3 bp hairpin and its adjacent single-stranded regions are part of the Region-20nt. The ribonucleic adenosine is unpaired and positioned opposite of the 3 bp hairpin.

After reselection, twenty-eight Zn-DNA clones were sequenced (FIG. 4). When compared with the parental wild type sequence (class I Zn-DNA), the reselected Zn-DNA contained point mutations mostly outside of Region-20nt. About one third of these sequences have a T → A mutation at position 73, converting the T̄T mismatch in the wild type sequence to a Watson-Crick base pair. In one fourth of the reselected DNAs, the 5 nucleotide single-stranded bulge of the three-way junction has the sequence 5'-ACGAA-3', corresponding to 5'-TCGAA-3' in the wild type. Clone #17 (named ZnR17) of the reselected Zn-DNA is most active under selection conditions (FIG. 4). Structural analysis of ZnR17 revealed two completed base-paired helices in the three-way junction. Therefore, it was engineered into a *trans*-cleaving deoxyribozyme by deleting the sequences outside of the three-way junction and the loop of the 8 bp hairpin. Such truncation resulted in two individual stands, which hybridize with each other through two 9-10 bp helices. The strand containing the single ribonucleotide residue (rA) is considered as the substrate (named 17DS), while the other strand as the enzyme (named 17E). The catalytic core, which was highly conserved during selection, consists of a 3 bp hairpin and a 5 nt single-stranded bulge (FIG. 5).

Although ZnR17 was selected in Zn^{2+}, it does not contain structure motifs that were discovered in several Zn(II)-binding RNA molecules (Ciesiolka *et al.*, 1995; Ciesiolka & Yarus, 1996). However, the conserved region of ZnR17 is very similar to that of the 8̄17 deoxyribozymes selected by Santoro and Joyce using Mg^{2+} as cofactor (Santoro & Joyce, 1997). The unpaired bulge region in the 8̄17 DNA enzyme has the sequence 5'-WCGR-3' or 5'-WCGAA-3' (W = A or T; R = A or G). The highest activity was observed with the sequence containing 5'-TCGAA-3'.

Among the Zn(II)-dependent deoxyribozymes we obtained after reselection, 85% of them fell within the 5'-WCGAA-3' regime (W = A or T). However, the sequence of the two double helices flanking the catalytic core is different between the $\bar{8}17$ (FIG. 6D) and the 17E deoxyribozymes (FIG. 6F), reflecting their different designs of the selection pool. Similar sequence motif was also observed in an RNA-cleaving deoxyribozyme (named Mg5) selected by Faulhammer and Famulok using 50 mM histidine and 0.5 mM Mg^{2+} as cofactors (Faulhammer & Famulok, 1997). The homologous region in 31 out of the 44 sequenced clones had the sequence 5'-$TX_1X_2X_3AGCY_1Y_2Y_3ACGAA$-3' (SEQ ID NO:9), falling within the WCGAA-3' regime. The authors predicted a secondary structure different from the $\bar{8}17$ or 17E motif based on chemical modification analysis. However, a structure containing a three-way junction similar to that of the 17E and $\bar{8}17$ deoxyribozymes is consistent with the chemical mapping results.

Sequence Alignment and Structure Analysis of Co-DNA

The sequences of the *cis*-cleaving deoxyribozyme selected in the presence of Co^{2+} are more diverse than the Zn-DNA. They can be divided into three major classes based on sequence similarity (FIG. 3). There is no consensus sequence region among different classes. The secondary structure of each sequence class of Co-DNA was predicted with *DNA mfold* program. The minimal conserved sequence motif of class I Co-DNA includes a bulged duplex. The cleavage site is within the 13 nt single-stranded bulge. A 4 bp hairpin is also highly conserved and linked to the bulged duplex through 3 unpaired nucleotides. The folding of the sequences outside of this minimal motif was highly variable and resulted in structures with a wide range of stabilization energy.

The class II Co-DNA contains a sequence region (5'-ACCCAAGAAGGGGTG-3' (SEQ ID NO:10)) that was also found in an RNA-cleaving deoxyribozyme (termed G3) selected by Geyer and Sen (1997) (FIG. 7A and 7B). The minimal motif predicted for class II Co-DNA shows similarity to that proposed for the G3 deoxyribozyme as well. The G3 deoxyribozyme was believed to be fully active in the absence of any divalent metal ions. Copious use of divalent metal chelating agents, such as EDTA, and accurate trace-metal analysis of the

reaction solutions were used to rule out the need of the G3 deoxyribozyme for contaminating levels of divalent metals. As mentioned earlier, the activity of class II Co-DNA was the same in buffer alone or with added Co^{2+} or Zn^{2+}, suggesting that this class of Co-DNA most likely contain the divalent metal-independent structure motif.

Effect of Metal Ions on the Activity of the *Cis*-cleaving Deoxyribozymes

ZnR17 and Co-18 were examined for their activity dependence on monovalent ions and divalent metal ions other than Zn^{2+} and Co^{2+}. In the presence of 1 mM EDTA and without added Zn^{2+} ions, no cleavage was observed with ZnR17 even after two days, strongly suggesting that divalent metal ions are required for the activity of ZnR17. Although the *cis*-cleaving Zn-DNA was selected in the presence of 500 mM NaCl, NaCl was actually inhibitory to enzymatic activity. With 0–2 M NaCl added to the reaction buffer (100 μM Zn^{2+}, 50 mM HEPES, pH 7.0), k_{obs} decreased with increasing NaCl concentration. The deleterious effect of NaCl was also manifested by lowered final percentage of cleavage products. For instance, only 50% of ZnR17 could be cleaved in the presence of 2 M NaCl even after long incubation times, while >95% of the DNA was cleavable in the absence of extra NaCl. Contrary to the Zn-DNA, the activity of Co-18 relies on NaCl and no cleavage was observed in the absence of NaCl. With 1 M NaCl, 8% of Co-18 molecules were cleaved within 5 min, while < 0.2% were cleaved in the absence of extra NaCl.

Although the deoxyribozymes were selected using either zinc or cobalt as cofactor, they are also active in other transition metal ions and in Pb^{2+}. The cleavage efficiency of ZnR17 followed the trend of $Pb^{2+} > Zn^{2+} > Mn^{2+} \sim Co^{2+} \sim Ca^{2+} > Cd^{2+} >> Ni^{2+} > Mg^{2+}$. It is noteworthy that the cleavage rate in Ca^{2+} was much higher than in Mg^{2+}, a similar trend was observed with the Mg5 deoxyribozyme. The order of Co-18 activity is as follow: $Zn^{2+} > Pb^{2+} \sim Co^{2+} > Ni^{2+} > Cd^{2+} \sim Mn^{2+} > Mg^{2+} \sim Ca^{2+}$. In general, both ZnR17 and Co-18 are more active in transition metal ions than in alkaline-earth metals, and higher activities were achieved with Pb^{2+}, Co^{2+} and Zn^{2+}. The preference of the selected deoxyribozymes for Co^{2+} and Zn^{2+} reflected their selection criteria. A similar trend ($Pb^{2+} > Zn^{2+} > Mn^{2+} > Mg^{2+}$) was also observed with all four RNA-cleaving deoxyribozymes selected in parallel by Breaker and Joyce

using one of the four metal ions (Pb^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}) as cofactor (1995). The proposed secondary structures of the deoxyribozymes selected in Pb^{2+} and Mg^{2+} have been reported (Breaker & Joyce, 1994, 1995). No structure similarity was observed between ZnR17 and those RNA-cleaving deoxyribozymes.

Summary

Using *in vitro* selection technique, several groups of RNA-cleaving deoxyribozymes were isolated using Zn^{2+} or Co^{2+} as cofactor. No common sequence or structural features were observed between the Co(II)-dependent and the Zn(II)-dependent deoxyribozymes, in spite of the chemical similarities between these two transition metal ions. The deoxyribozymes selected in Zn^{2+} share a common motif with the $\tilde{8}17$ and the Mg5 deoxyribozymes isolated under different conditions, including the use of different cofactors. Both the Co-DNA and the Zn-DNA exhibited higher activity in the presence of transition metal ions than in alkaline earth metal ions, which are the most commonly adopted metal cofactors by naturally occurring ribozymes.

Example 3 - Deoxyribozyme based biosensor for Pb^{2+} detection

This Example describes a particle-based biosensor for the detection of Pb^{2+}. The biosensor utilizes the deoxyribozyme developed in Example 2 (termed 17E) combined with particle technology to allow quantitative and real time measurements of catalytic activity. Because catalytic activity is dependent on Pb^{2+}, the biosensor provides real-time, quantitative, and sensitive measurements of Pb^{2+} concentrations.

DNA/RNA chimera oligonucleotide cleavable substrate.

The oligonucleotides were purchased from Integrated DNA Technology, Inc, Coralville, IA. The cleavable substrate is a 44 base DNA/RNA chimera oligonucleotide with the sequence 5'-TGTCAACTCGTG-ACTCACTATrAGGAAGAGATG-TGTCAACTCGTG-3'(SEQ ID NO:85)), in which rA represents a ribonucleotide adenosine. The cleavable substrate strand is derived from the substrate 17DS 5'-ACTCACTATrAGGAAGAGATG-3' (SEQ ID NO:2) (See Example 2). 17DS has been extended on both the 3' and 5' ends

for twelve bases, which act as "sticky end" for annealing to a complementary DNA strand on the gold particles.

Thiol-modified DNA gold particles.

Thiol-modified oligonucleotides were purchased from Integrated DNA Technology, Inc, Coralville, IA. A thiol-modified 12mer DNA primer, 5'-SH-$(CH_2)_6$-CACGAGTTGACA-3'(SEQ ID NO:86) was attached to the 13 nm diameter gold particles (produced as in Example 1) by adding the 3.6 μM thiol-modified DNA to the gold colloid. The solution was allowed to sit for 24 hrs, and then diluted to give a final concentration of 100 mM NaCl and 25 mM Tris-acetate buffer, pH 7.2. After another 24hrs, the DNA attached gold particles were centrifuged at 14,000 rpm for one hour. The supernatant was removed and the concentrated gold particles washed with 100 mM NaCl buffer. After another centrifuge at 14,000 rpm, the particles were stored in 300 mM NaCl, 25mM Tris-acetate buffer.

Preparation of agglutinated gold particles.

500 pmol cleavable substrate and 1,000 pmol *trans*-cleaving deoxyribozyme (17E) were added to DNA linked gold particles (absorption of 2.2 at 522 nm) to a final volume of 3 mL. The sample was buffered at pH 7.2 using 25mM Tris-acetate (TA) and 500mM NaCl. The mixture was heated to 70°C and allowed to cool slowly to room temperature. A dark purple precipitation was formed at the bottom of the tube. This is due to the aggregation of 13 nm gold particles by the DNA linker. After centrifugation for one minute, the precipitation were collected and resuspended in 4mL of 300 mM NaCl, 25mM TA buffer pH7.2.

The *trans*-cleaving deoxyribozyme is required for aggregate formation. Without the deoxyribozyme, the DNA modified gold particles and cleavable substrate did not aggregate. A deoxyribozyme titration experiment was performed to determine the optimum ratio of deoxyribozyme and cleavable substrate. The extinction at 522 nm was shown to represent the relative amount of separated gold particles while the extinction at 700 nm was shown to represent the relative amount of gold particle aggregates. Hence, the ratio of extinction at 700 nm to 522 nm was used to monitor the degree of aggregation. This procedure minimized artifacts due to concentration

differences. Aggregation increased with deoxyribozyme concentration until the ratio of deoxyribozyme to μ reached one.

UV-vis extinction spectrum of separated and aggregated gold particles

The UV-vis extinction spectrum of separated and aggregated gold particles was determined as follows. Gold particles were prepared as in Example 1. Aggregated gold particles were prepared as in Example 3. UV-vis extinction spectra were obtained by monitoring the surface plasmon band of gold nanoparticles. For an non-aggregated particles, the extinction peak is 522 nm and decreases rapidly at longer wavelengths, giving the suspension a deep red color. When the particles are in the aggregated state, the extinction peak shifts to a longer wavelength and falls off less quickly. A suspension of aggregated particles develops a purple color.

Example 4 - Color change of the Pb^{2+} sensor in the presence of Pb^{2+} due to enzymatic substrate cleavage.

To verify that the cleavage is carried out by the deoxyribozyme 17E and using Pb^{2+} only as a cofactor, a control experiment replacing 17E with an inactive deoxyribozyme 17E-C: 5'-CATCTCTTCCCCGAGCCGGTCGAAATAGTGAGT-3' (SEQ ID NO:87) was performed. Previous kinetics assays have shown that the activity of 17E was complete lost when the G•T wobble pair was replaced by a G•C Watson-Crick base pair. (Santoro, S.W. & Joyce, G.F. (1997); Faulhammer, D. & Famulok, M. (1997).) 17E-C is an inactive version of 17E by changing the important T base to a C base. Since only one base is changed, the structure of the substrate-17E duplex is very similar to the duplex formed by the substrate and 17E-C.

Aggregated particles were prepared as in Example 3. In addition, aggregated particles containing the inactive deoxyribozyme, 17E-C instead of the active deoxyribozyme were also prepared using the same method. A Pb^{2+} assay was then performed as follows. 48 μL of particle suspension was pipetted into microcentrifuge tubes. 2 μL of a 5 μMPb^{2+} standard ($Pb(OAc)_2$ (Aldrich, St. Louis, MO) in water) was added to each tube and the tubes heated above 50 °C for 5 minutes. The melting temperature of the aggregated particles was previously determined by measuring the extinction at 260nm. The DNA linked gold particles

showed a sharp melting temperature (46 °C). Thus, heating the aggregated particles to a temperature higher than 50 °C resulted in the fully melting of the aggregate.

The tubes were allowed to cool naturally to room temperature and UV-vis spectra measured. When active deoxyribozyme was present, cleavage of the substrate was observed at 5 μM Pb^{2+}. However, with inactive deoxyribozyme, cleavage was not observed, even in the presence of 5 μM Pb^{2+}.

Example 5 - Semi-quantitative and quantitative DNA Pb^{2+} Assays.

Semi-quantitative protocol – An assay was performed as in the previous example. After the tubes cooled at room temperature, a 10 μL sample was removed and used to spot on an alumina TLC plate (Analtech, Newark, DE). A color progression from purple (no lead present) to red (high lead concentration) was observed. Hence, the Pb^{2+} concentration in a sample may be determined by comparing the color obtained with the sample with that obtained for standard Pb^{2+} solutions or with a standard color chart.

Quantitative protocol – After the tubes cooled at room temperature UV-vis spectra were obtained using a Hewlett-Packard 8453 spectrophotometer. To eliminate the effect of concentration, the ratio of extinction at 522 nm and 700 nm was used to monitor the degree of aggregation of particles. These two wavelengths were chosen to represent the relative amount of aggregated and free gold particles.

Example 6 - Detection range of a DNA Pb^{2+} Assay.

Agglutinated gold particles were prepared as in Example 3 except that a 1 to 1 ratio of deoxyribozyme to substrate was used. A Pb^{2+} assay was performed as in Example 4. Pb^{2+} standards in the range 0 - 5μM were tested and the results visualized on a TLC plate and by UV-vis extinction spectroscopy. Pb^{2+} standards in the range from 250 nM to 1 μM were distinguishable visually on the plate. A second assay was performed using a 1 to 20 mixture of active and inactive deoxyribozyme. Here, the assay the detection region was from 10 μM - 200 μM. Hence, by simply varying the amount of active deoxyribozyme, the detection range of the assay may be varied to

adopt to different detection requirements. In addition to visual detection, results were determined quantitatively using the ratio of extinction at 522 nm and 700 nm.

Example 7 - Cross-reactivity characteristics of a DNA Pb^{2+} Assay.

Standards containing 5 μM Pb^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, $Zn^{2+}$and Cd^{2+} ions in water were prepared by dissolving $Pb(OAc)_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$, $ZnCl_2$, and $Cd(OAc)_2$ (Aldrich, St. Louis, MO) in water. A Pb^{2+} assay was performed as in Example 4 to determine the cross-reactivity of 5 μM Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, $Zn^{2+}$and Cd^{2+}. The standards containing these ions were tested along with the standard containing 5 μM Pb^{2+}. These divalent metal ions are chosen for assay because they have shown relative high activities as a metal cofactor for enzymatic cleavage. At 5 μM, all seven metal ions give a blue color on a TLC plate. However, 0.5 μM Pb^{2+} developed a purple color. In addition, results were determined quantitatively using the ratio of extinction at 522 nm and 700 nm. The results obtained with Mg^{2+}, Co^{2+}, and Zn^{2+} are shown in Table 3. Of the ions tested, Zn^{2+} and Co^{2+} showed the highest cross reactivity.

Table 3 – Detection of Pb^{2+} in the presence of Mg^{2+}, Co^{2+}, and Zn^{2+} ions.

Sample Name	Extinction at 522 nm	Extinction at 700 nm	E522/E700
pure water	0.91982	0.43821	2.099
1uM Pb^{2+}	1.0839	0.17929	6.045
1uM Pb^{2+}+1uM Co^{2+}	1.0844	0.1582	6.854
1uM Pb^{2+}+1uM Mg^{2+}	1.0911	0.1739	6.274
1uM Pb^{2+}+1uM Zn^{2+}	1.1691	0.12972	9.0124

Example 8 - Room temperature DNA Pb^{2+} Assay. (Prophetic)

This example describes a room temperatue DNA Pb^{2+} Assay. Thiol-modified oligonucleotides may be purchased from Integrated DNA Technology, Inc., Coralville, IA. The thiol-modified 12mer DNA primer, 5'-SH-$(CH_2)_6$-CACGAGTTGACA-3'(SEQ ID NO:88) is used to modify gold particles as in Example 3. In addition, a second type to modified gold particles are produced using the same primer sequence but with a 3'-thiol linkage. Agglutinated gold particles are

prepared using the method described in Example 3 except that a 1:1 mixture of 3'-thiol and 5'-thiol DNA linked particles is used.

A Pb^{2+} assay is then performed as follows. 48 μL of particle suspension is pipetted into microcentrifuge tubes. 2 μL of a Pb^{2+} standard is added to each tube and the tubes incubated at room temperature. The UV-vis extinction spectra are then determined. A breakdown of aggregation is observed with increasing Pb^{2+} concentration. Thus, the modified assay does not require the heating and cooling steps of the unmodified assay.

Example 9 - Detection of Pb2+ in leaded paint.

The DNA based sensor was used to detect Pb^{2+} in leaded paint. Leaded paint containing various concentrations of lead was simulated by adding basic lead carbonate 0%, 0.5%, 1%, 2%, 3%, 5% 10% by weight of $PbCO_3 \bullet Pb(OH)_2$ (Aldrich, St. Louis, MO) to a commercial white paint containing a TiO_2 pigment. (Exterior white paint, Glidden, Cleveland, OH). The leaded paint was dissolved in 10% HOAc. After diluting this solution 150000 times in water, a Pb^{2+} assay was performed. Results were determined in a modified semi-quantitative protocol. Briefly, 2 μL of 50 times concentrated aggregated particles (prepared as in Example 3) were added to 98 μL of the diluted paint solution. This mixture was then heated to 50 °C for 5 minutes and allowed to cool slowly to room temperature. A 10μL sample was spotted onto a TLC plate. A color pattern developed on the TLC plate showing a color transition between 2 and 3 % indicating that the Pb^{2+} concentrations in the original solutions is approximately 500 nM. With the knowledge of the times of dilution, the concentration of the original soaking solution can be calculated.

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CLAIMS

1. A method of detecting the presence of an ion, comprising:

(a) forming a mixture comprising an aggregate and a sample; and

(b) detecting deaggregation of the aggregate;

wherein the aggregate comprises a nucleic acid enzyme, a substrate and particles, the nucleic acid enzyme is dependent on the ion to cause cleavage of the substrate, and the substrate comprises nucleotides.

2. The method of claim 1, wherein the ion is AsO_4^{3-}.

3. The method of claim 1, wherein the ion is selected from the group consisting of K^+, Na^+, Li^+, Tl^+, NH_4^+, Ag^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Hg^{2+}, Hg_2^{2+}, Pt^{2+}, Ra^{2+}, Ba^{2+}, UO_2^{2+}, Sr^{2+}, Co^{3+}, Cr^{3+}, Ln^{3+}, Ce^{4+}, Cr^{6+}, spermine and spermidine.

4. The method of claim 1, wherein the ion comprises a member selected from the group consisting of K(I), Na(I), Li(I), Tl(I), Ag(I), Hg(I), Mg(II), Ca(II), Mn(II), Co(II), Ni(II), Zn(II), Cd(II), Pb(II), Hg(II), Pt(II), Ra(II), Ba(II), Sr(II), Co(III), Cr(III), Ln(III), Ce(IV), Cr(VI) and U(VI).

5. The method of claim 1, wherein the sample comprises a plurality of different ions.

6. The method of claim 5, wherein the ion is Pb^{2+}.

7. The method of claim 1, wherein the ion is a metal ion.

8. The method of claim 7, wherein the metal ion is Ag^+.

9. The method of claim 7, wherein the metal ion is Hg^{2+}.

10. The method of claim 1, wherein the sample comprises a bodily fluid.

11. The method of claim 10, wherein the bodily fluid is blood.

12. The method of claim 1, wherein the particles are gold particles.

13. The method of claim 12, wherein an oligonucleotide is linked to the particles.

14. The method of claim 13, wherein the oligonucleotide is linked by thiol coupling.

15. The method of claim 14 wherein a second oligonucleotide is linked to the particles.

16. The method of claim 1, wherein the particles comprise at least two sets, wherein a first oligonucleotide is linked to a first set of particles and a second oligonucleotide is linked to a second set of particles and, wherein the base sequence of the first oligonucleotide is different from the base sequence of the second oligonucleotide.

17. The method of claim 1, wherein the nucleic acid enzyme comprises a ribozyme.

18. The method of claim 1, wherein the nucleic acid enzyme comprises a deoxyribozyme.

19. The method of claim 18, wherein the nucleic acid enzyme comprises SEQ ID NO:1.

20. The method of claim 1, wherein the substrate comprises SEQ ID NO:2.

21. The method of claim 1, wherein the detecting deaggregation of the aggregate comprises detecting a color change.

22. The method of claim 21, wherein the color change may be detected visually.

23. A composition comprising a nucleic acid enzyme, a substrate and particles, wherein the nucleic acid enzyme is dependent on an ion to cause cleavage of the substrate, and the substrate comprises nucleotides.

24. The composition of claim 23, wherein the particles are gold particles.

25. The composition of claim 23, wherein the particles comprise at least two sets, wherein a first oligonucleotide is linked to a first set of particles and a second oligonucleotide is linked to a second set of particles and, wherein the base sequence of the first oligonucleotide is different from the base sequence of the second oligonucleotide.

26. The composition of claim 23, wherein the substrate comprises a first portion and a second portion, wherein a cleavable portion of the substrate separates the first portion and the second portion.

27. The composition of claim 26, wherein the cleavable portion of the substrate is cleavable by the nucleic acid enzyme in the presence of the ion.

28. The composition of claim 26, wherein the first and second portions of the cleavable substrate comprise oligonucleotides.

29. The composition of claim 28, wherein the oligonucleotide on the first portion of the cleavable substrate is complementary to the oligonucleotide on the first set of particles and the oligonucleotide on the second portion of the cleavable substrate is complementary to the oligonucleotide on the second set of particles.

30. The composition of claim 29, wherein the cleavable substrate and the particles are linked by complementary oligonucleotide binding.

31. The composition of claim 30, wherein the particles form an aggregate.

32. A kit for detecting an ion, comprising:

(a) a cleavable substrate;

(b) particles; and

(c) a nucleic acid enzyme,

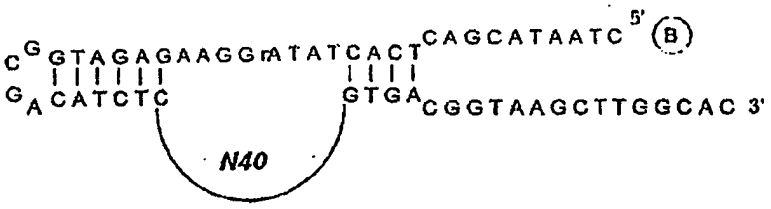
wherein, the nucleic acid enzyme is dependent on the ion to cause cleavage of the substrate, and the substrate comprises nucleotides.

33. The kit of claim 32, wherein the cleavable substrate, particles and nucleic acid enzyme are supplied in separate containers.

34. The kit of claim 32, wherein the cleavable substrate, particles and nucleic acid enzyme are supplied combined.

35. The kit of claim 34, wherein the cleavable substrate, particles and nucleic acid enzyme are supplied as an aggregate.

36. The kit of claim 32, further comprising a solution having a known concentration of the ion.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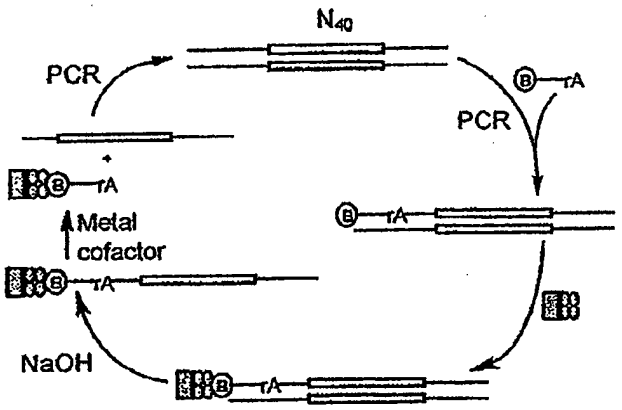
CATCTC
GTGA
CGGTAAGCTTGGCAC 3'
N40

FIG. 1A


N40
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B rA
PCR
B rA
Metal
cofactor
B rA
B rA
NaOH
B rA

FIG. 1B

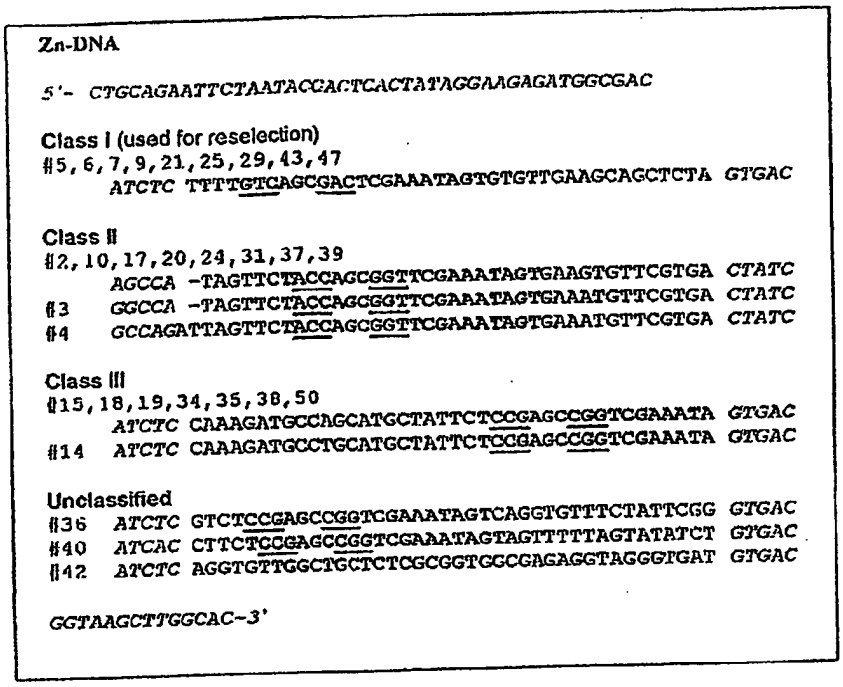
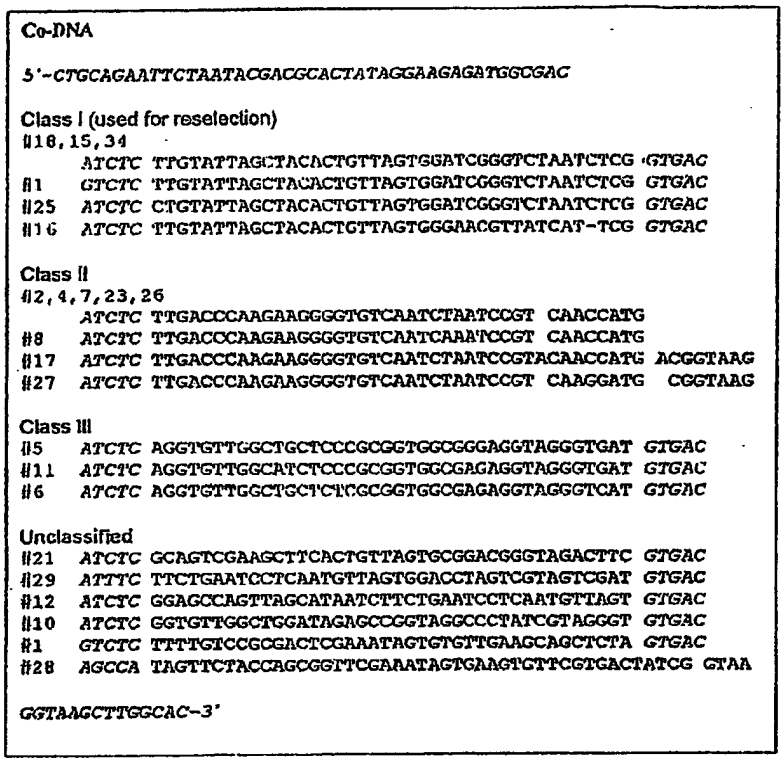

Zn-DNA
5'- CTGCAGAATTCTAATACGACTCACTATAGGAAGAGATGGCGAC
Class I (used for reselection)
#5,6,7,9,21,25,29,43,47
ATCTC TTTTGTCAGCGACTCGAAATAGTGTGTTGAAGCAGCTCTA GTGAC
Class II
#2,10,17,20,24,31,37,39
AGCCA -TAGTTCTACCAGCGGTTCGAAATAGTGAAGTGTTCGTGA CTATC
#3 GGCCA -TAGTTCTACCAGCGGTTCGAAATAGTGAAATGTTCGTGA CTATC
#4 GCCAGATTAGTTCTACCAGCGGTTCGAAATAGTGAAATGTTCGTGA CTATC
Class III
#15,18,19,34,35,38,50
ATCTC CAAAGATGCCAGCATGCTATTCTCCGAGCCGGTCGAAATA GTGAC
#14 ATCTC CAAAGATGCCTGCATGCTATTCTCCGAGCCGGTCGAAATA GTGAC
Unclassified
#36 ATCTC GTCTCCGAGCCGGTCGAAATAGTCAGGTGTTTCTATTCGG GTGAC
#40 ATCAC CTTCTCCGAGCCGGTCGAAATAGTAGTTTTTAGTATATCT GTGAC
#42 ATCTC AGGTGTTGGCTGCTCTCGCGGTGGCGAGAGGTAGGGTGAT GTGAC
GGTAAGCTTGGCAC-3'

FIG. 2


Co-DNA
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Class I (used for reselection)
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#1 GTCTC TTGTATTAGCTACACTGTTAGTGGATCGGGTCTAATCTCG GTGAC
#25 ATCTC CTGTATTAGCTACACTGTTAGTGGATCGGGTCTAATCTCG GTGAC
#16 ATCTC TTGTATTAGCTACACTGTTAGTGGGAACGTTATCAT-TCG GTGAC
Class II
#2,4,7,23,26
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#8 ATCTC TTGACCCAAGAAGGGGTGTCAATCAAATCCGT CAACCATG
#17 ATCTC TTGACCCAAGAAGGGGTGTCAATCTAATCCGTACAACCATG ACGGTAAG
#27 ATCTC TTGACCCAAGAAGGGGTGTCAATCTAATCCGT CAAGGATG CGGTAAG
Class III
#5 ATCTC AGGTGTTGGCTGCTCCCGCGGTGGCGGGAGGTAGGGTGAT GTGAC
#11 ATCTC AGGTGTTGGCATCTCCCGCGGTGGCGAGAGGTAGGGTGAT GTGAC
#6 ATCTC AGGTGTTGGCTGCTCTCGCGGTGGCGAGAGGTAGGGTCAT GTGAC
Unclassified
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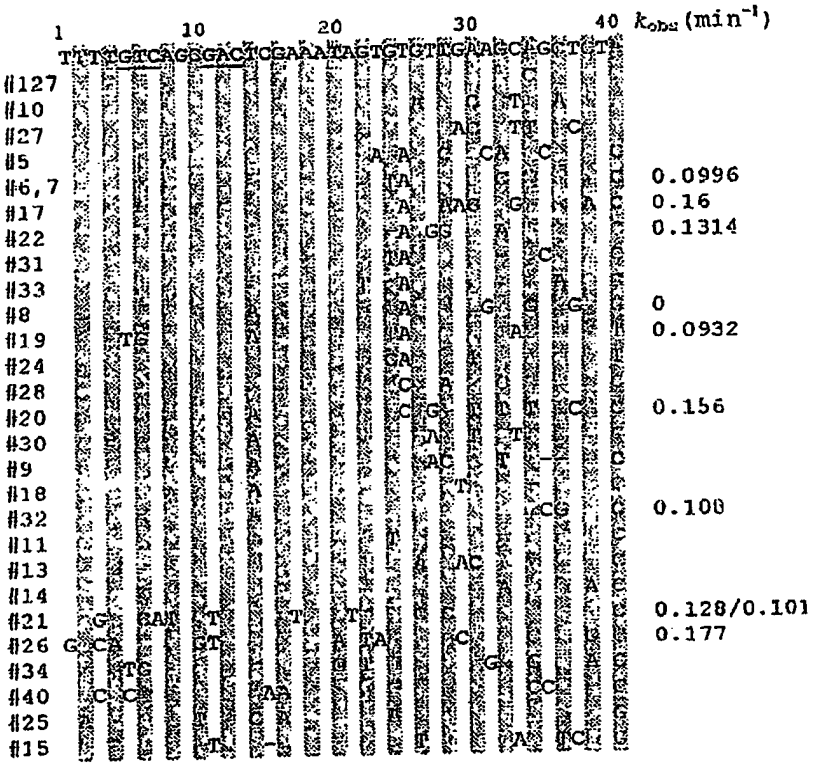
FIG. 3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#27
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#17
#22
#31
#33
#8
#19
#24
#28
#20
#30
#9
#18
#32
#11
#13
#14
#21
#26
#34
#40
#25
#15
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0.100
0.128/0.101
0.177

FIG. 4

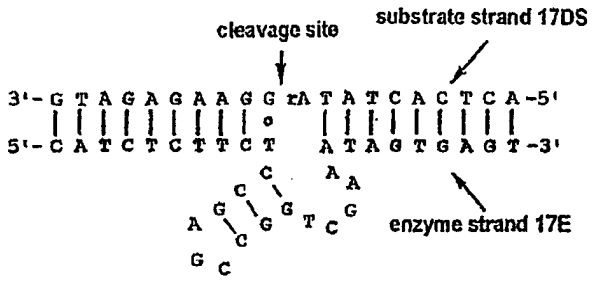

cleavage site
substrate strand 17DS
3'-GTAGAGAAGGrATATCACTCA-5'
5'-CATCTCTTCT ATAGTGAGT-3'
enzyme strand 17E

FIG. 5

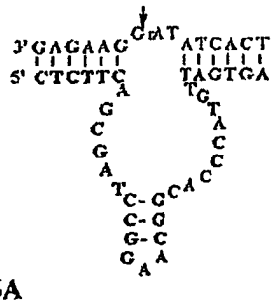


FIG. 6A

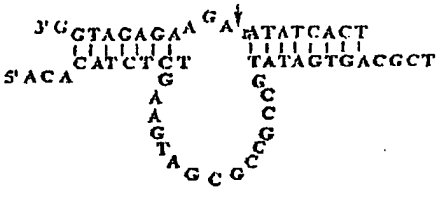


FIG. 6B

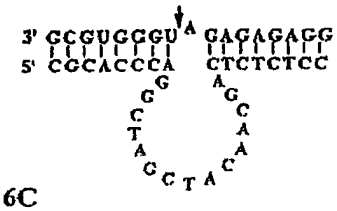


FIG. 6C

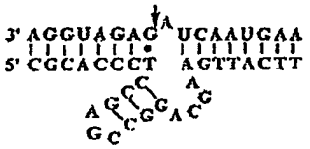


FIG. 6D

FIG. 6E

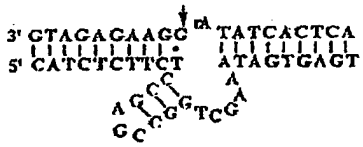


FIG. 6F

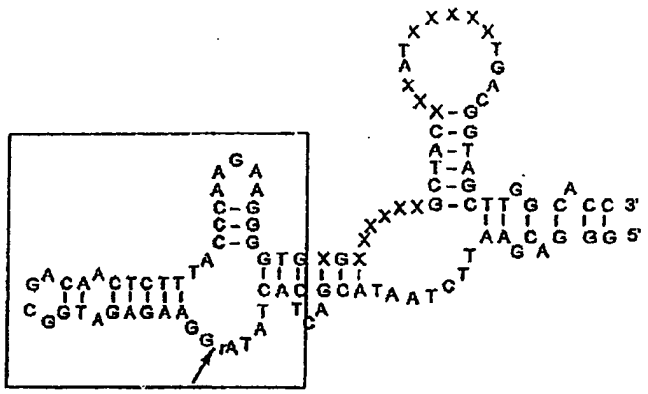


FIG. 7A

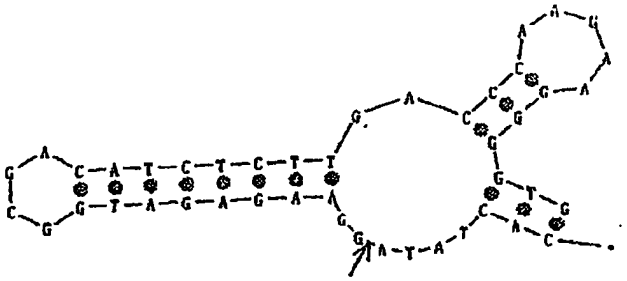
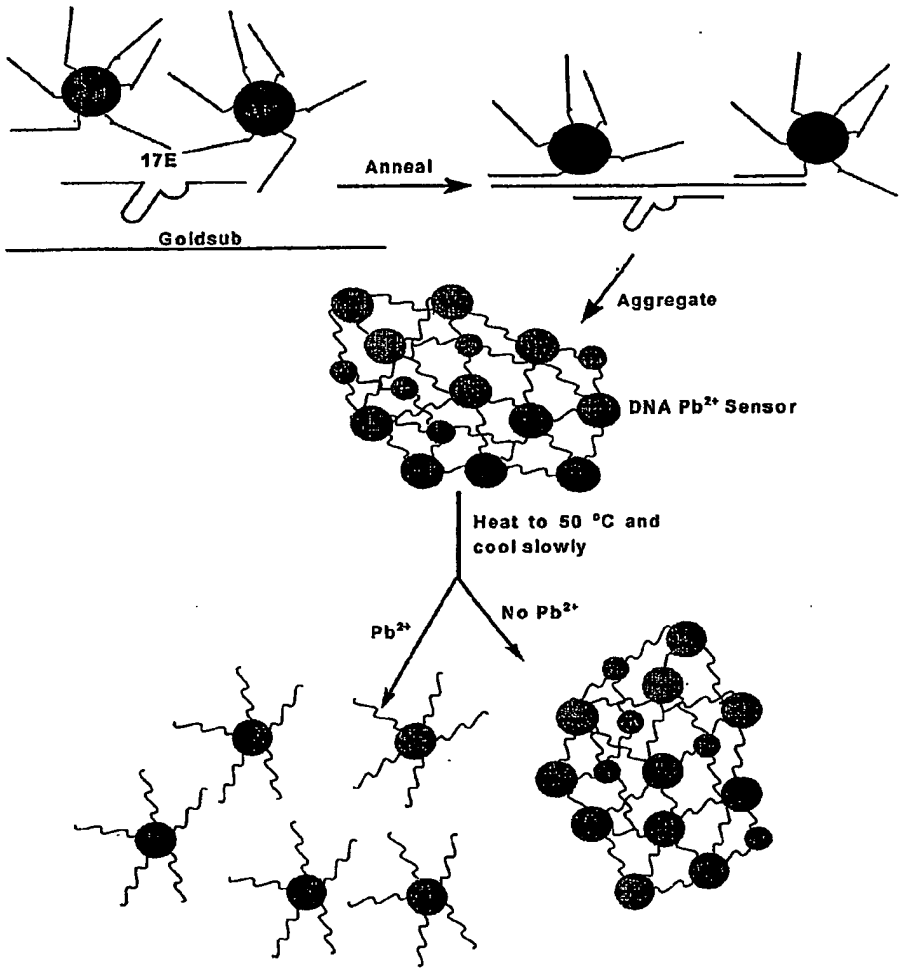


FIG. 7B



Goldsub – cleavable substrate . Au – Gold particle. 17E – nucleic acid enzyme.

Figure 8

SEQUENCE LISTING

<110> LU, YI
LIU, Juewen

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<130> 10322/44

<140> UNDETERMINED
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<160> 84

<170> PatentIn Ver. 2.1

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Co-DNA

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<223> Description of Artificial Sequence: Zn-DNA

<400> 43
ttttgtcagc gactcgaaat agtgtgttga agcagctcta 40

<210> 44
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 44
ttttgtcagc gactcgaaat agtgtgttga agccgctcta 40

<210> 45
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 45
ttttgtcagc gactcgaaat agtgtattgc agtagatcta 40

<210> 46
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 46
ttttgtcagc gactcgaaat agtgtgttac agttgcccta 40

<210> 47
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 47
ttttgtcagc gactcgaaat agagagtcga cacacctctc 40

<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 48
ttttgtcagc gactcgaaat agttagttga accagctctc 40

<210> 49
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 49
ttttgtcagc gactcgaaat agtgagtaag aggagctatc 40

<210> 50
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 50
ttttgtcagc gactcgaaat agtgagggga aacagctctc 40

<210> 51
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 51
ttttgtcagc gactcgaaat agttagttga acacctctc 39

<210> 52
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 52

<210> 53
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 53
ttttgtcagc gacacgaaat agtgagttga ggcggcgctg 40

<210> 54
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 54
tttttgcagc gacacgaaat agttagttga agaagctctt 40

<210> 55
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 55
ttttgtcagc gactcgaaat agtcagttgt agcagctctt 40

<210> 56
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 56
ttttgtcagc gactcgaaat agtgcgtaga accagctctc 40

<210> 57
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 57
ttttgtcagc gacacgaaat agtgcggtgt atctgccctc 40

<210> 58
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 58
ttttgtcagc gacacgaaat agtgtgatgt agtagctctc 40

<210> 59
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 59
ttttgtcagc gacacgaaat agtgtgacga atcatctc 38

<210> 60
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 60
ttttgtcagc gacacgaaat agtgtgttta agcgctctc 39

<210> 61
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 61
ttttgtcagc gacacgaaat agtgtgttga agcacgtctc 40

<210> 62
<211> 40
<212> DNA
<213> Artificial Sequence

<223> Description of Artificial Sequence: Zn-DNA

<400> 62
ttttgtcagc gactcgaaat agtttgttga agcagctctc 40

<210> 63
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 63
ttttgtcagc gactcgaaat agtgtattac agcagctctc 40

<210> 64
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 64
ttttgtcagc gactcgaaat agtgtgttga aacagctatc 40

<210> 65
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 65
ttgtgcatgc tactcgtaat tgtgtctcga agcagctctc 40

<210> 66
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 66
gtcagtcagg tactcgaaaa atagtgttca agccgctgtc 40

<210> 67
<211> 40

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 67
tttttgcagc gactcgaaag attgtgttga ggcggctatc 40

<210> 68
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 68
ttctctcagc gactaaaaat agtgtgttga agcccctctc 40

<210> 69
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 69
tattgtcagt gacccaaaat agtatgttga agcagctctg 40

<210> 70
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Zn-DNA

<400> 70
ttttgtcagc tactgaaata gtgttttgaa gaagtcctg 39

<210> 71
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic chimeric substrate

<220>
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric substrate

<400> 71
tcactatagg aagag 15

<210> 72
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic chimeric substrate

<220>
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric substrate

<400> 72
ctcttcagcg atccggaacg gcacccatgt tagtga 36

<210> 73
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic chimeric substrate

<220>
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric substrate

<400> 73
tcactataag aagagatgg 19

<210> 74
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic chimeric substrate

<220>
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric substrate

<400> 74
acacatctct gaagtagcgc cgccgtatag tgacgct 37

<210> 75
<211> 17

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic chimeric substrate

<220>
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric substrate

<400> 75 17
ggagagagau gggugcg

<210> 76
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic chimeric substrate

<220>
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric substrate

<400> 76 31
cgcacccagg ctagctacaa cgactctctc c

<210> 77
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic chimeric substrate

<220>
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric substrate

<400> 77 17
aaguaacuag agaugga

<210> 78
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic chimeric substrate

<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric substrate

<400> 78
cgcaccctcc gagccggacg aagttactt 29

<210> 79
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic chimeric substrate

<220>
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric substrate

<400> 79
ctcactatag gaagagatg 19

<210> 80
<211> 41
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic chimeric substrate

<220>
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric substrate

<400> 80
catctcttaa cggggctgtg cggctaggaa gtaatagtga g 41

<210> 81
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic chimeric substrate

<220>
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric substrate

<400> 81
actcactata ggaagagatg 20

<210> 82
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic chimeric substrate

<220>
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric substrate

<400> 82
catctcttct ccgagccggt cgaaatagtg agt 33

<210> 83
<211> 107
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Predicted secondary structure of the G3 deoxyribozyme

<220>
<221> modified_base
<222> (67)
<223> variable nucleotide

<220>
<221> modified_base
<222> (69)..(74)
<223> variable nucleotide

<220>
<221> modified_base
<222> (80)..(82)
<223> variable nucleotide

<220>
<221> modified_base
<222> (85)..(89)
<223> variable nucleotide

<400> 83
gggacgaatt ctaatacgac tcactatagg aagagatggc gacaactctt tacccaagaa 60
ggggtgngnn nnnngctacn nnatnnnnnt gacggtagct tggcacc 107

<210> 84
<211> 45
<212> DNA
<213> Artificial Sequence

<220>

<400> 84
cactatagga agagatggcg acatctcttg acccaagaag gggtg 45

<400> 85

5'-TGTCAACTCGTG-ACTCACTATrAGGAAGAGATG-TGTCAACTCGTG-3' (SEQ ID NO:85)

<400> 86

5'-SH-$(CH_2)_6$-CACGAGTTGACA-3' (SEQ ID NO:86)

<400> 87

5'-CATCTCTTCCCCGAGCCGGTCGAAATAGTGAGT-3' (SEQ ID NO:87)

<400> 88

5'-SH-$(CH_2)_6$-CACGAGTTGACA-3' (SEQ ID NO:88)